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The accurate diagnosis of leishmaniasis requires the development of specific and sensitive reagents, particularly recombinant antigens or peptides. The best example is the development of the recombinant antigen K39, specific for the *L. donovani* complex, that allows for rapid and accurate diagnosis of visceral leishmaniasis to a degree of sensitivity and specificity higher than direct parasite observation. The syndrome known as viscerotropic leishmaniasis (VTL) presents special diagnostic challenges. The number of infected individuals remains unknown at this time due to the extreme difficulty in isolation of the parasite combined with a lack of common symptoms to aid in diagnosis. We have previously isolated and characterized antigens reactive with sera from *L. tropica* infected Desert Storm soldiers. Effort has been to further evaluate the diagnostic capabilities of a previously identified *L. tropica* antigen, through testing and optimization of synthetic peptides derived from this antigen. Effort has also been directed at recovery of additional immunodominant *Leishmania* antigens, specifically potent T cell antigens secreted by *Leishmania* promastigotes. Strategies to identify promastigote secreted antigens have resulted in the isolation of several novel antigens. These antigens are currently being expressed and evaluated both for cellular responses and serological reactivity in individuals infected with *Leishmania*.

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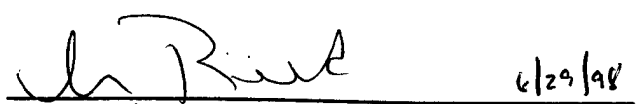

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## INTRODUCTION

Work performed over the first twelve month period focused on three areas. First, continued work was performed on a peptide derived from the repeated portion of *L. tropica* antigen, Lt-1 (1). ELISA based assays were optimized, and data was generated to better determine the specificity and sensitivity of the peptide, using additional sera from non-deployed disease-free individuals, gulf war veterans, and cutaneous *L. tropica* patients. Second, additional peptides derived from the Lt-1 repeat region and the repeat region from an additional *L. tropica* antigen, Lt-4, were evaluated as additional diagnostic components in an ELISA based assay using normal and gulf war VTL sera.

We have shown that recombinant antigen based assays offer increased sensitivity and specificity in diagnostic assays (1, 2, 4-6). Therefore, recent effort has been made to identify the antigenic components secreted or shed by *Leishmania* promastigotes. We have concentrated on expression cloning using sera from animals immunized with secreted protein from *Leishmania* promastigotes. We have identified several antigens of interest from *L. tropica, L. major*, and *L. chagasi* libraries. These antigens are currently being expressed and evaluated in cellular assays to determine their stimulatory potential and possible inclusion in a confirmatory diagnostic cellular based assay.

## BODY

The emphasis of work over the first twelve month period included the optimization of the reactive epitope contained within the thirty-three amino acid repeat present in the Lt-1 antigen. Previous work with a subclone, Lt-1r, that encoded approximately one and one-third repeat, demonstrated the reactive epitope was present within this forty-five amino acid stretch. Because of continuing specificity problems with the Lt-1r recombinant, peptides were synthesized and evaluated. Three overlapping 20 amino acid peptides (Lt1r1, Lt1r2, Lt1r3) covering the 33 amino acid repeat failed to demonstrate reactivity with VTL patient sera.

Four larger peptides were synthesized representing overlapping portions of the first two repeats present in the Lt-1 recombinant. These were Lt1r4 (45 amino acid), Lt1r5 (33 amino acid), Lt1r6 (50 amino acids), and Lt1r7 (66 amino acids). These four peptides were evaluated on both VTL patient sera and normal sera in an ELISA based assay. Lt1r4 and Lt1r7 demonstrated increased reactivities with VTL patient sera compared to that seen with normal sera. Both of the peptides were superior to the Lt1r recombinant, with enhanced specificity. Lt1r4 demonstrated better specificity than Lt1r7, likely due to a single degeneracy present in the second repeat of the Lt1r7 peptide, but not present in the Lt1r4 peptide.

ELISA assays using Lt1r4 were optimized for antigen level, sera dilution, and reporting system. An increased level of sensitivity was achieved using a reporting system of Protein A-biotin, Streptavidin-HRP (horseradish peroxidase), and TMB (3, 3', 5, 5'-tetramethylbenzidine). Sensitivity and specificity of the Lt1r4 peptide was further assessed by using additional sera from non-deployed disease-free individuals, gulf war veterans, and Turkish cutaneous *L. tropica* patients. In an ELISA based assay (Fig. 1), a comparison of *L. tropica* infected cutaneous patient sera to a panel of normals yielded a sensitivity of 50% (6/12) and a specificity of 98.5% (67/68) using a cut-off established at normal mean + three standard deviations.

A panel of sera was received from a collaboration with Dr. Mike Riscoe (Portland Veterans Affairs Medical Center) involving the program project "Unexplained Illness among Veterans of the Persian Gulf Conflict". This panel, including sera from both symptomatic and asymptomatic individuals, was tested for reactivity with the Lt1r4 peptide (Fig. 2). A surprisingly high number of sera (10/110) had reactivities above the normal distribution (Fig. 1), but it is unclear at this time whether these reactivities are indicative of an infection by *L. tropica*. Additional information obtained on this group in the course of the study may offer some clarification and contribute to the generation of an appropriate group of control sera useful in the evaluation of additional candidate diagnostic antigens.

A titration of sera from four of the high Army samples, one Turkish cutaneous leishmaniasis, and three normals (including the one normal found above mean + 3 SD) is shown in Figure 3, clearly indicating specific differential reactivity with the Lt1r4 peptide.

An additional serological epitope had been previously localized to a 39 amino acid repeat present in two overlapping *L. tropica* antigens, Lt-4 and Lt-5. A peptide approach was emphasized to maximize specificity. Two peptides were synthesized representing overlapping portions of the first two repeats present in the Lt-4 recombinant. These were Lt4pep1 (39 amino acids) and Lt4pep2 (78 amino acids). These two peptides were evaluated in an ELISA based assay and were not significantly more reactive with VTL patient sera than with normal sera (data not shown).

Due to the likelihood that multiple serological epitopes will be necessary to achieve an acceptable level of sensitivity, we have cloned an additional *L. tropica* gene by expression cloning using mouse sera immunized with promastigote secreted protein mixture. The resulting *L. tropica* gene cloned shows a high degree of homology with a family of thiol-specific antioxidant proteins. The gene has been re-engineered for expression (addition of histidine tag) and recombinant protein purified. The recombinant is currently being evaluated with VTL and normal sera in an ELISA based format. The recombinant will also be tested in cellular assays using peripheral blood mononuclear cells from *L. tropica* infected cutaneous leishmaniasis patients from Saudi Arabia, recently received as part of a collaborative effort with Dr. Hashim Ghalib (King Saud University, Abha, Saudi Arabia).

An additional method used to isolate immunodominant antigens secreted or shed by *Leishmania* promastigotes has involved expression cloning of leishmania genomic and cDNA libraries with rabbit sera immunized with the promastigote secreted mixture. This has resulted in the isolation of several novel *Leishmania* genes. Genomic southern analysis has shown that all three genes are highly conserved among the different *Leishmania* species including *L. tropica* (data not shown). Recombinant antigens encoded by the three genes have been expressed and purified, and will be tested for serological reactivity and stimulatory activity in cellular assays.

Important progress has also been made towards the two major limitations in the development of a diagnostic for *L. tropica*. The first limitation is the lack of availability of a proper panel of control sera-from individuals deployed to the Gulf during Operation Desert Storm but had no chance of exposure to *L. tropica*. A collaboration with Dr. Mike Riscoe at Portland Veterans Affairs Medical Center involving Gulf War Veterans may provide a number of sera from individuals with limited or incomplete deployment. The second limitation involves the need for additional sera from individuals with confirmed *L. tropica* infection. A longstanding collaborator, Dr. Hashim Ghalib, has recently re-located to Abha, Saudi Arabia, an area endemic for *L. tropica*. Through this collaboration, we have recently received both sera and PBMC from *L. tropica* infected individuals with cutaneous leishmaniasis. An additional collaboration with Dr. Nevin Turgay (Ege University Medical School, Izmir, Turkey), has provided a panel of sera from regional cases of cutaneous leishmaniasis caused by *L. tropica*.

The second twelve month period has focused on the cloning and characterization of novel leishmanial antigen genes. Over the period, 10 new antigens have been cloned and characterized. These are summarized in Table 1. As the original aims of the proposal were to define antigens with both serological and T cell reactivity, we have focused in both areas. One of the antigens we have cloned is a novel protein of *L. major* with a molecular weight of 22.1 kDa (7). The predicted amino acid sequence of this clone exhibited significant sequence homology to eukaryotic thiol-specific antioxidant (TSA) proteins, thus we have designated this protein *L. major* TSA. Southern blot hybridization analyses indicate that the TSA gene is a multicopy gene in all *Leishmania* species analyzed, and that additional gene copies are present in two species causing cutaneous leishmaniasis (*L. major* and *L. tropica*). Northern blot analyses indicate that TSA is expressed constitutively in *L. major* promastigotes and amastigotes. Recombinant TSA protein containing an amino-terminal 6 histidine tag was expressed in *E. coli* using the pET17b system and was purified to homogeneity by affinity chromatography. The recombinant protein elicited both T cell proliferation and IFN gamma production in leishmaniasis patient PBMC. In separate studies, we have immunized BALB/c mice with recombinant TSA in IFA which resulted in the development of strong humoral and cellular immune responses to both recombinant TSA and *L. major* promastigote lysate. Together these data

suggest that the TSA protein may be useful as a diagnostic antigen for leishmaniasis. In further studies, we have characterized T cell responses to LeIF (2). LeIF is a leishmanial protein that was first described as an antigen that strongly induced IFN-γ in leishmaniasis patients at least in part by its ability to directly stimulate monocytes/macrophages to make IL-12. Lymphocytes from leishmaniasis patients stimulated with LeIF produced high amounts of IFN-γ and no detectable IL-4. In addition, LeIF was shown to decrease SLA induced IL-4 production. These data demonstrate that LeIF may be a potent diagnostic antigen for leishmaniasis. We do not know, in the absence of further samples from *L. tropica* infected military personnel, whether these antigens could serve as diagnostic candidates using cellular response assays in vitro or in the intradermal skin test.

Studies are continuing to characterize T cell reactivity to the novel antigens discovered in the project period. We have begun testing the antigens in serological assays, emphasizing sera from the limited Gulf War panel that was made available to us. The results are summarized in Fig. 4, which shows serological data from 8 novel recombinant proteins that are shared between *L. major* and *L. tropica*. The antigens were tested on a panel of sera that included 24 samples from Gulf War military personnel previously tested positive for the Lt1r-4 antigen, as well as normal controls. Several of the antigens, including 1E6, 2A10, 4A5, and 8G3 were found to detect specific antibody in at least some of the individuals in the military personnel panel over reactivity detected in the normal sera. These data indicate that, with fine epitope mapping and assay optimization, it should be possible to use recombinant leishmanial proteins to detect leishmania specific antibody, even in individuals with relatively low antibody titers.

## CONCLUSIONS

We have fulfilled the aims of the proposal which were to clone and characterize new leishmanial antigens with diagnostic potential. Several potent antigens capable of detecting leishmanial infection have been cloned and are now available. The lack of availability of increased numbers of patients with parasite positive *L. tropica* infection and VTL syndrome has hampered our studies, but we have used a variety of other patient types, including cutaneous *L. tropica* infection, to pursue our studies.

The focus of work in the first year included the further evaluation of known *L. tropica* antigens reactive with *L. tropica* infected military personnel. Persistent reactivity with normal sera led to the evaluation of peptides designed against repeated portions of the antigens. Evaluation of multiple peptides derived from the 33 amino acid repeat present in Lt-1, identified the best candidate, Lt1r4, which was optimized in an ELISA based assay. Use of the peptide did increase specificity to the current estimate of 98%, but sensitivity was not increased, estimated at approximately 50% in both cutaneous leishmaniasis and viscerotropic leishmaniasis. Although it is possible that the sensitivity could be increased by further generation and characterization of peptide variants, it is likely more fruitful to identify additional antigens that can complement the existing peptide. To that end, peptides derived from another *L. tropica* antigen known to react with VTL patient sera (rLt4 ) were generated and evaluated. These peptides failed to show increased reactivity with VTL sera. An additional recombinant reactive with VTL patient sera (Lt3) is currently being engineered for expression.

Another source of new antigens is the secreted or shed material from promastigotes, which contains potent T cell antigens. In the past year, effort has been made to identify these antigens, resulting in the recovery of a number of candidate clones, all of which have now been expressed and purified in milligram quantities. We have now expressed and characterized recombinant protein from several novel leishmanial antigens cloned during the course of the project period. These antigens have been evaluated on the limited panel of sera made available to us during the funding period. As a result of this project, multiple candidate antigens are available for clinical and laboratory investigations focusing on VTL.

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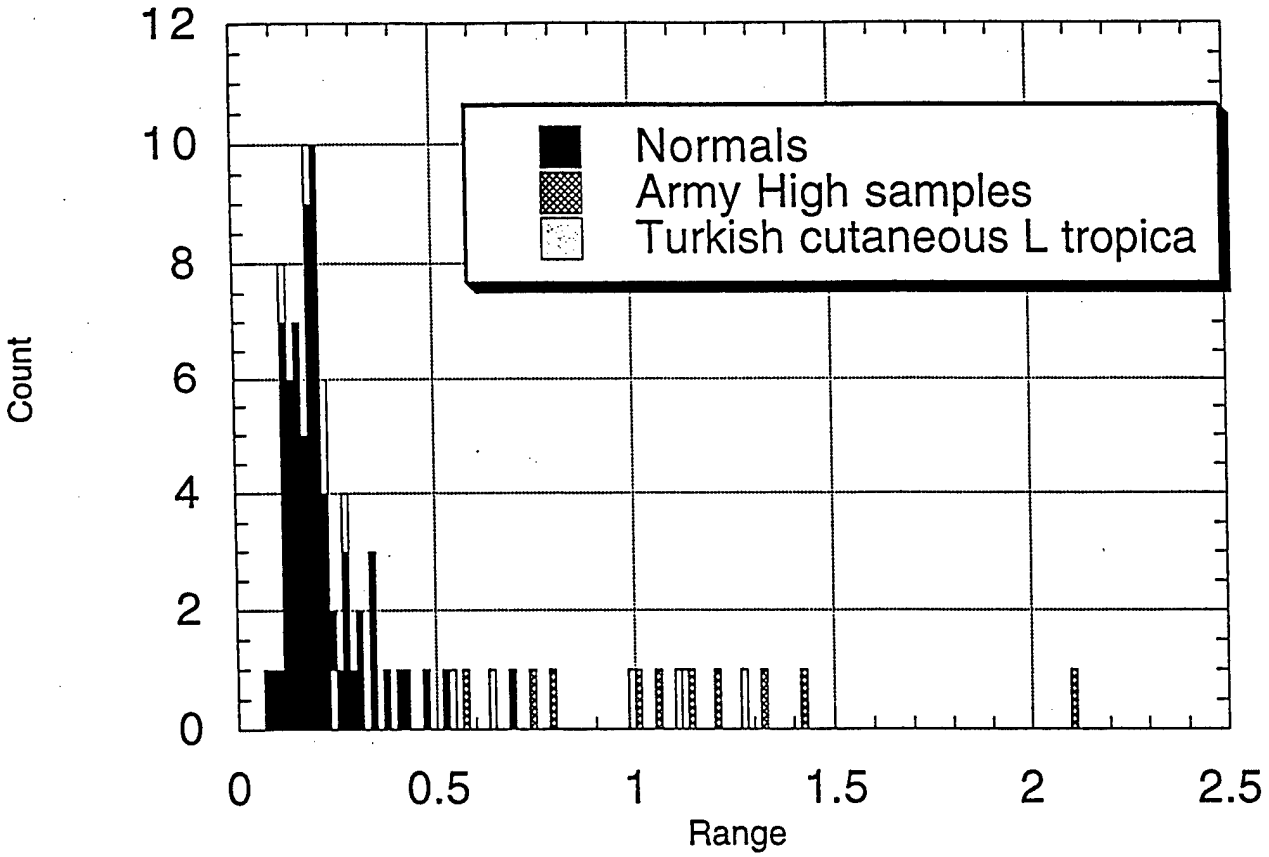


Figure 1. Distribution of reactivity of Lt1r4 peptide with patient and normal sera. Sera from normal individuals (n=68), *L. tropica* cutaneous leishmaniasis patients (n=12) and elevated peptide reactive Gulf War Veterans (n=10) used at 1:50 dilution in an ELISA based assay. Lt1r4 peptide was used at 1 ug /well. Bioin-Protein A (1:5000) and streptavidin-HRP (1:8000) were used and the substrated used was TMB.

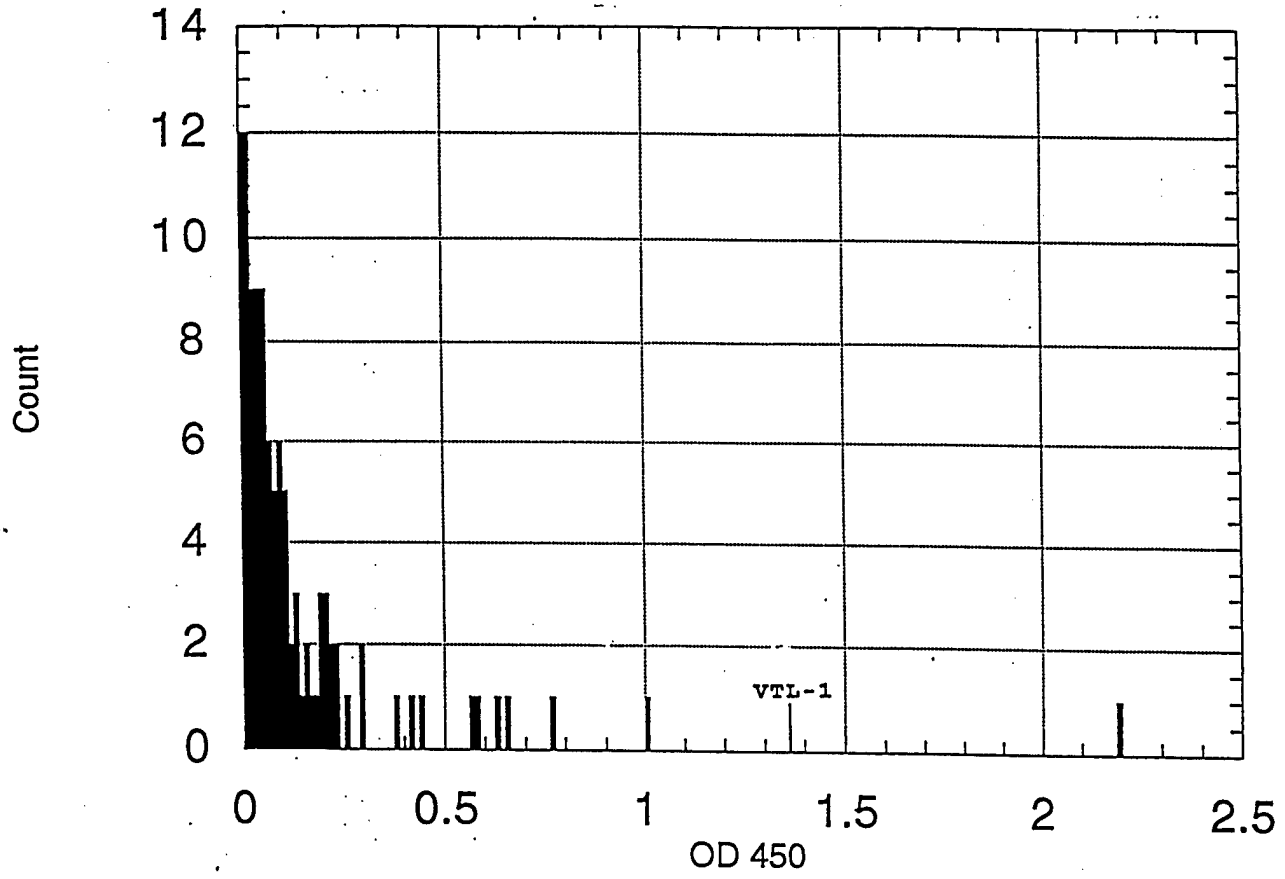


Figure 2. Distribution of reactivity of Lt1r4 peptide with Gulf War Veteran sera. Sera from Gulf War Veterans (n=110) used at 1:50 dilution in an ELISA based assay. Lt1r4 peptide was used at 1 ug /well. Bioin-Protein A (1:5000) and streptavidin-HRP (1:8000) were used and the substrated used was TMB. Reactivity of confirmed viscerotropic leishmaniasis sera (VTL-1) shown.

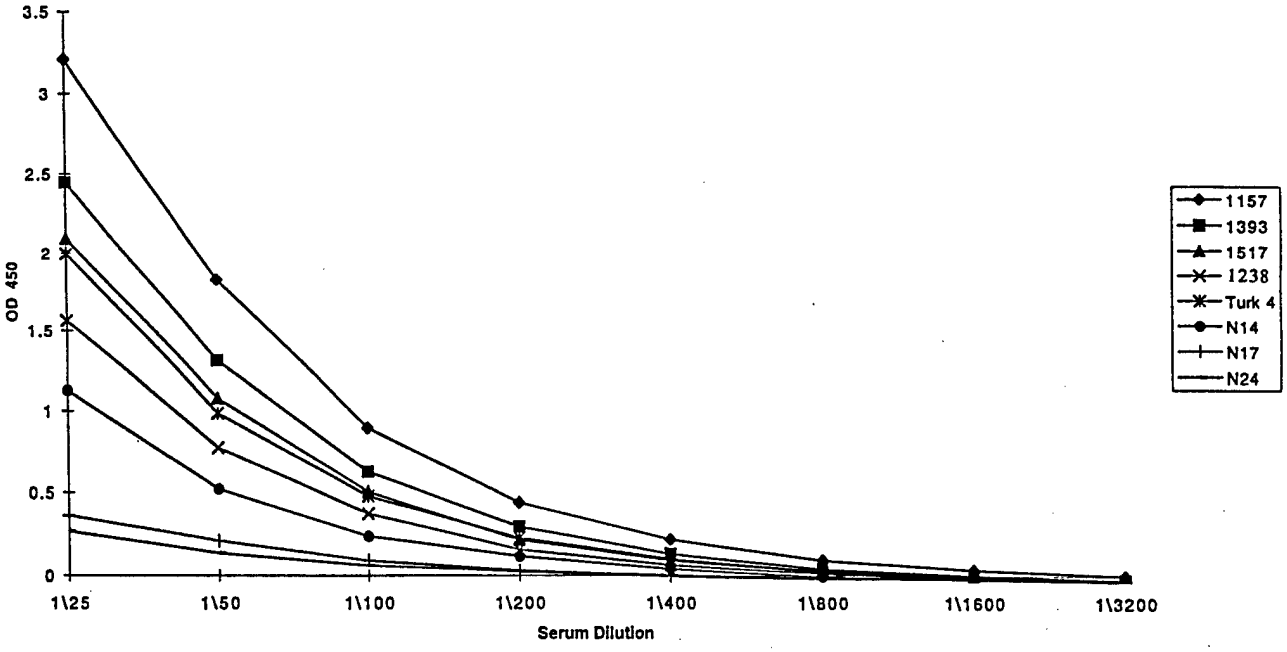


Figure 3. Titration of Sera . Titration of four reactive sera from Gulf War Veterans (1157, 1393, 1517, 1238), one reactive Turkish *L. tropica* cutaneous sera, and three normal sera (N14-normal sera sample above mean + 3 SD cut-off, N17, N24). Lt1r4 peptide was used at 1 ug /well. Bioin-Protein A (1:5000) and streptavidin-HRP (1:8000) were used and the substrated used was TMB.

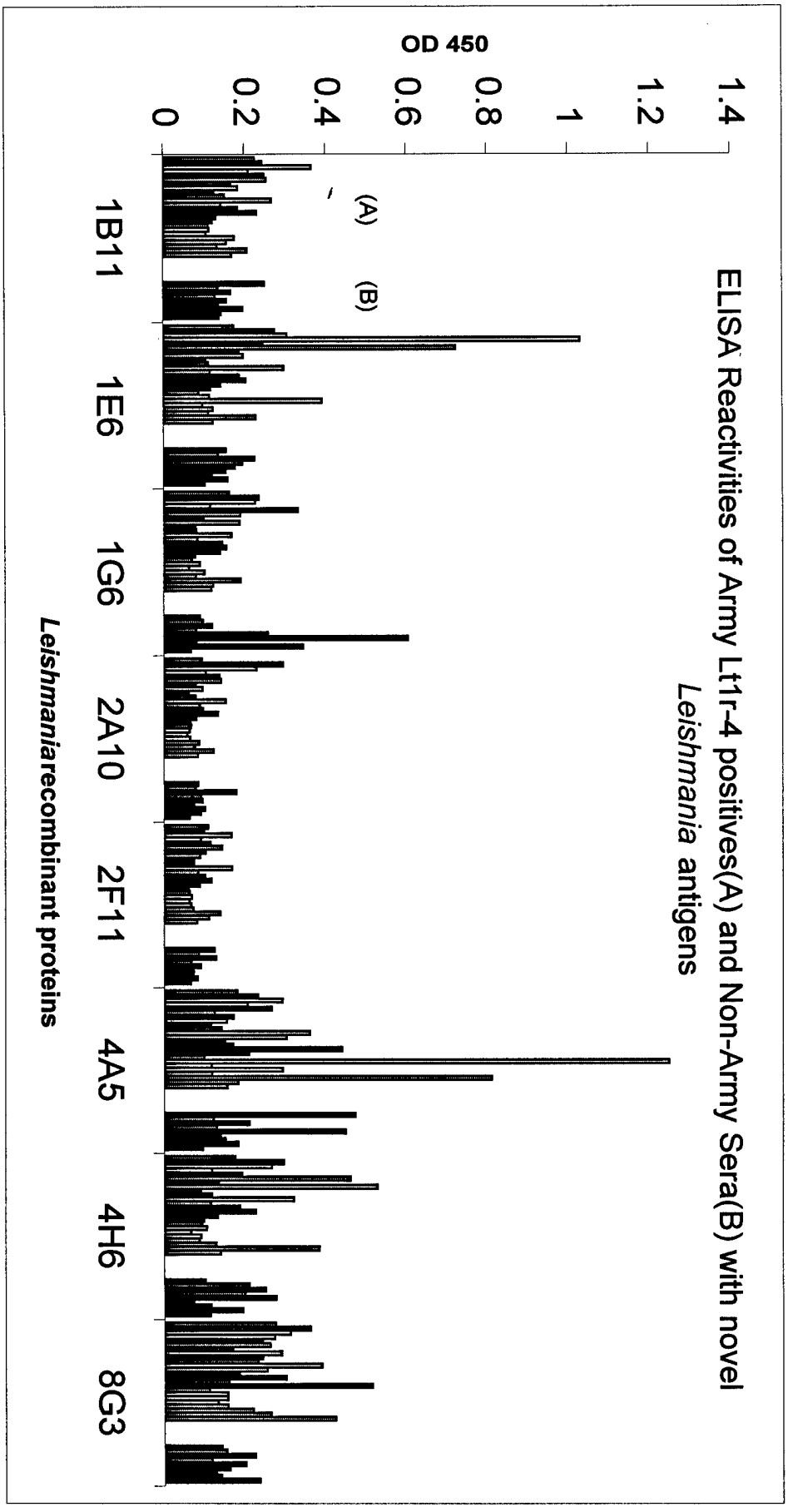
ELISA Reactivities of Army Lt1r-4 positives(A) and Non-Army Sera(B) with novel
Leishmania antigens
OD 450
1.4
1.2
1
0.8
0.6
0.4
0.2
0
(A)
(B)
1B11
1E6
1G6
2A10
2F11
4A5
4H6
8G3
Leishmaniarecombinant proteins

FIGURE 4

## Table 1. Novel *Leishmania* Secretory Protein Antigens

| Name | Homology |
|---|---|
| 8G3 | malate dehydrogenase |
| 1E6 | p45 |
| 1G6 | histone H2b |
| 4A5 | p21 |
| 2A10 | α-tubulin |
| 2F11 | EiF2A |
| 1B11 | ribosomal S4 |
| 4H6 | β-tubulin |

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**Bibliography:**

No publications or meeting abstracts have been published.

**Personnel receiving pay from the negotiated effort:**

Antonio Campos-Neto
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Tricia Martin